Reference: *Biol. Bull.*, **143**: 525–547. (December, 1972)

STUDIES ON *FES*, A MUTATION AFFECTING CYSTOCYTE CYTOKINESIS, IN *DROSOPHILIA MELANOGASTER*

JOAN HEWLETT JOHNSON AND ROBERT C. KING

Department of Biological Sciences, Northwestern University, Evanston, Illinois 60201

Unlike somatic cells, the insect oocyte must contain sufficient nutrient reserves to maintain the potential organism during embryogenesis, because most insect embryos have no means for obtaining exogenous, organic raw materials. Furthermore, since little transcription of RNA takes place along DNA cistrons during mitosis, and since a period of rapid mitosis occurs early in embryogenesis, a mechanism must also exist for loading the unfertilized egg with the long-lived messenger RNAs, ribosomes, and transfer RNAs that are required to synthesize the proteins utilized during this early period of development. Higher insects have solved these problems by evolving methods for providing (1) endopolyploid cells which synthesize the required compounds and (2) a system of canals through which these products can be exported to the oocyte. In the fruitfly, *Drosophila melanogaster*, for example, the females are characterized by ovarioles that contain egg chambers in which the oocyte is one member of a cluster of 16 interconnected cells (King, 1970). The egg and the interconnected nurse cells are descendants of a single cell, the germarial "cystoblast." The interconnected cells formed by a division of a cystoblast are called "cystocytes." It is within the germarial portion of the ovariole that the consecutive mitoses occur that produce each cystocyte cluster, and it is here also that each cluster becomes enveloped by profollicle cells. The major growth of each egg chamber is completed in the more posterior portion of the ovariole (the vitellarium). Here the nurse cells undergo a series of endomitotic DNA replications and transfer their cytoplasm to the oocyte.

Females of *Drosophila melanogaster* homozygous for the autosomal recessive gene *fes* are rendered sterile because they produce "ovarian tumors" instead of eggs (see King, 1969a, for review). Each tumor in the vitellarium is composed of hundreds or thousands of cells that resemble cystocytes in that they are similar in size, are mitotically active, and are sometimes interconnected. Ovaries of *fes* genotype become "tumorous" even when they are transplanted into the abdomens of wild type females, and wild type ovaries transplanted into *fes* females do not become tumorous (King and Bodenstein, 1965). Therefore there is no evidence for diffusible tumorigenic agents as initiating factors in the development of these tumors.

Since *fes* cystocytes frequently continue dividing rather than differentiating, we concluded that the primary effect of the mutation is an alteration of the pattern of cystocyte divisions in the germarium. Normally a branched chain of sixteen cells is generated, and the characteristic pattern of connections depends on the position of the interconnecting canals. In the germarium these "ring canals" are too small to be seen with the light microscope. Therefore a *fes* germarium was reconstructed from a series of composite electron micrographs made from serial sections in order

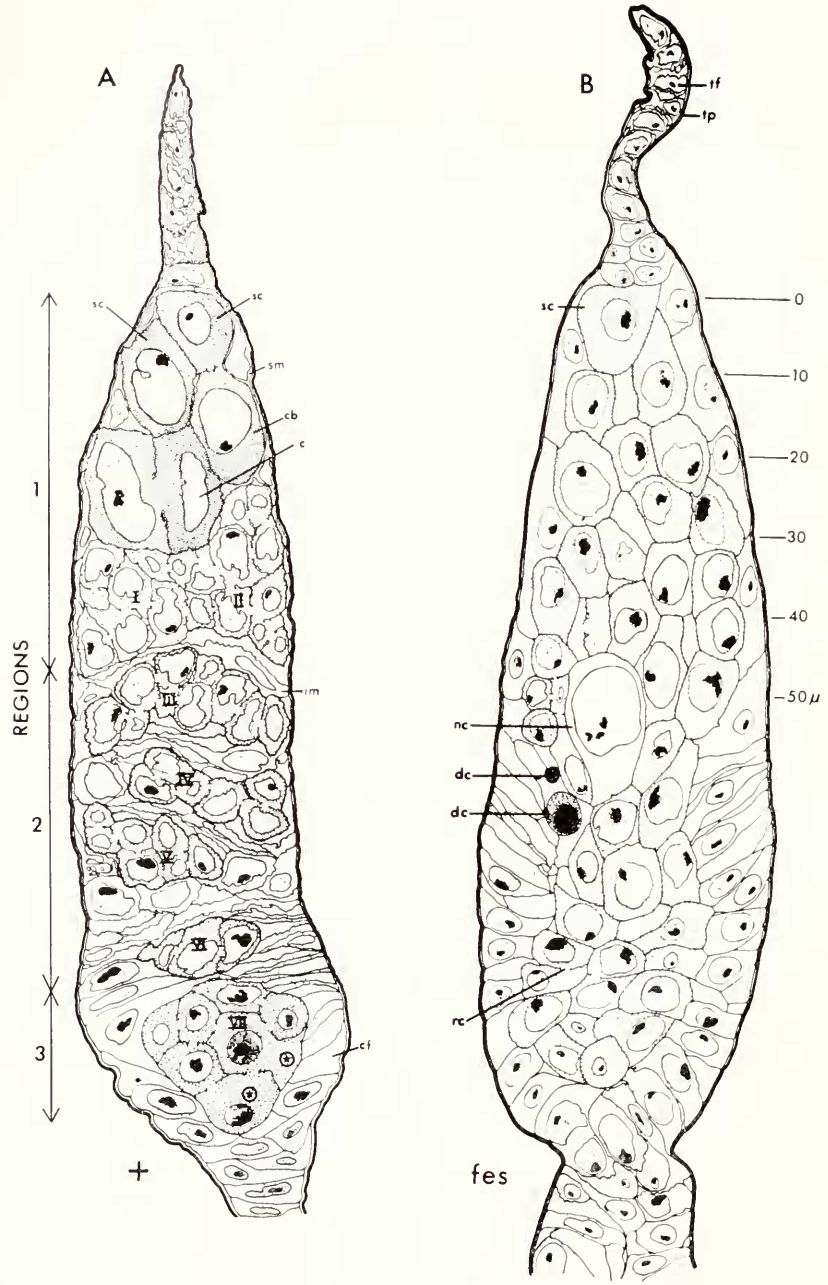

FIGURE 1.

to compare the interconnections of *fes* cystocytes with the normal pattern. This reconstruction provided a detailed, but static, picture of the contents of one germarium.

In order to study the dynamics of cell division in the germarium, the mitotic figures in hundreds of *fes* and wild type germaria were observed in Feulgen-stained whole mounts. The low frequency of metaphase figures observed made it desirable to investigate germaria, in which the divisions produced over a period of several hours were accumulated. The injection of flies with the mitotic poison, colchicine, produced the desired effect. The light microscopic study provided data on the dynamics of cell division in the germarium, and it also allowed us to determine whether or not the *fes* germarium studied with the electron microscope was typical.

Materials and Methods

Electron microscopy

Females of *Drosophila melanogaster* of genotype *S fes* + + *Alt lt*/+ *fes dp*^{tw}*Sp* + + were raised at 25° C on David's medium (David, 1962). See Lindsley and Grell (1968) for a description of the mutations used. Although the *fes* mutation has been renamed *fs(2)B* by Lindsley and Grell, the more familiar designation *fes* will be used throughout this paper. Homozygous *fes* ovaries were dissected in *Drosophila* physiological saline (the recipe is given on p. 142 of Butterworth, Bodenstein and King, 1965), and fixed for one hour at room temperature in Palade's veronal acetate buffer containing 1% osmium, and 1% potassium dichromate (Eakin and Westfall, 1962). The tissue was dehydrated through a series of aqueous ethanol solutions, transferred to propylene oxide and finally to a mixture of Epon and Araldite (Mollenhauer, 1964). Polymerization was carried out in a 60° C oven for three days.

Serial longitudinal sections were made of an entire *fes* germarium. Silver sections were cut on an LKB Ultrotome III with a section collecting side arm. The sections were mounted in groups of four on single hole grids coated with Formvar and carbon. The sections were stained for five minutes with lead citrate (Reynolds, 1963) and for fifteen minutes in saturated, aqueous uranyl acetate.

Every four section was photographed at a magnification of 4400 on a Hiatchi HS-8 electron microscope operated at 50 kV. Each of the 700 negatives obtained was enlarged 2.5 times, and the overlapping prints were assembled into series of composite electron micrographs. The cellular and nuclear membranes and the ring canals in each composite were traced on Kodak diffusion sheets. Once the tracings were stacked in order and viewed simultaneously, it became possible to follow and record the interconnections of all the cells.

Figure 1. (A.) A diagram of a median sagittal section through the germarium of a wild type *Drosophila melanogaster*. Roman numerals refer to sixteen cell clusters. The pro-oocytes are starred. This figure is adapted from Figure 1 of Koch and King (1966). (B.) A diagram of a median sagittal section through a germarium of a homozygous *fes* female. Mesodermal cells are drawn with pale stippling. Compare with Figure 1A, and see the text for further discussion. Abbreviations are: c, cystocyte; cb, cystoblast; cf, cuboidal follicle cell; dc, degenerating cell; im, invasive mesodermal cell; nc, nurse cell; rc, ring canal; sc, stem line oogonium; sm, squamous mesodermal cell; tf, terminal filament; tp, tunica propria.

Colchicine studies

Oregon R wild type and *fes* flies were reared for their entire life cycle at either 25° or 18° C in incubators with alternating twelve hour periods of light and darkness. Forty-eight hours after eclosion, the females were anesthetized with carbon dioxide and injected with a freshly prepared aqueous solution of colchicine (Carolina Biological Supply Co.). The injection was made using drawn-out capillaries for needles. Each injection contained 2–4 lambdas of fluid, enough to swell the abdomen slightly. Since injections of similar volumes of water had no detectable effect on mitosis, uninjected flies taken from the same cultures were used as controls. Flies were returned to the incubator in which they had been raised for the interval between injection and sacrifice.

The flies were chilled and then dissected in *Drosophila* physiological saline. The ovaries were prepared as Feulgen-stained whole mounts (see King, Burnett and Staley, 1957, page 242 for procedure). The slides were examined with a Wild M 20 research microscope at a magnification of 1250. The images of germaria were traced using a drawing tube. The drawings were calibrated with a stage micrometer. All measurements of the positions of cells or clusters were made with reference to the base of the terminal filament.

The effective concentration of colchicine for studying ovaries was determined using Oregon R females raised at 25° C. The concentration was varied from 1×10^{-2} to 1×10^{-7} M. Flies were sacrificed four hours after the injection. One hundred to one hundred fifty germaria were examined in each determination. Colchicine at a concentration of 1×10^{-4} M produced the maximum number of poisoned metaphases. A colchicine-metaphase (C-metaphase) is easily recognized because the affected chromosomes are shorter and more tightly coiled than in a normal metaphase.

In a similar study Oregon R females were injected with a 1×10^{-4} M colchicine and returned to the 25° C incubator for one to ten hours before being sacrificed. Again each determination was made utilizing one hundred to one hundred fifty germaria. Maximum values were obtained by 4 hours. Most metaphases from samples taken at 1, 2, and 3 hours were not C-metaphases; whereas all figures from $3\frac{1}{2}$ to 5 hours were C-metaphases. Later samples showed both types of metaphases. The duration of colchicine effects in cystocytes is relatively brief, but follicle cells are affected for as long as ten hours. The combination of 1×10^{-4} M colchicine, injected four hours prior to sacrificing the fly was judged optimal, and this regimen was used subsequently on Oregon R and *fes* females raised at 18° and 25° C.

Results and Conclusions

The ultrastructure of the fes *germarium*

The *fes* germarium that was reconstructed was sectioned slightly tangential to the longitudinal axis. A true longitudinal section was therefore drawn using tracings of cells seen in several composites made from the serial sections. The result, presented as Figure 1B, shows the general arrangement of the follicle cells and some of the tightly packed cystocytes. The mutant germarium should be compared to the normal germarium illustrated in Figure 1A.

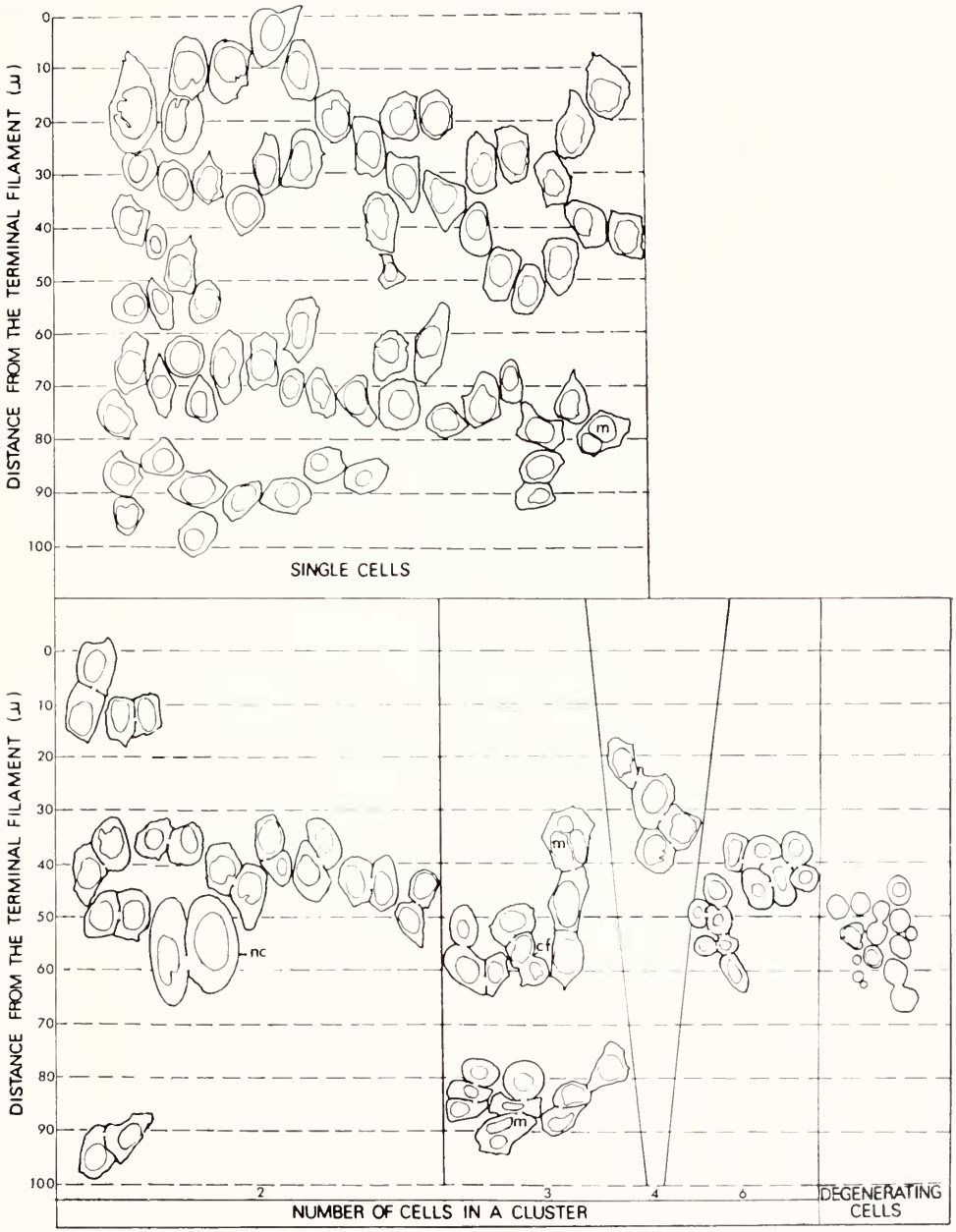

FIGURE 2. A diagram of the contents of a *fes* germarium based on serial electron micrographs. The cells are grouped as single cells; clusters of two, three, four, and six cells; and degenerating cells. Within each group, the cells are arranged with respect to their distance from the terminal filament. Each cell is represented by a tracing made of the electron micrograph showing the maximal cross sectional area of the cells. All ring canals are shown. Abbreviations are: cf, probable cleavage furrow; m, multinucleate cell; nc, nurse cell.

The position and interconnections of each cell in the germarium are shown in Figure 2. The maximal cross-sectional area of each cell, and all ring canals are illustrated in this diagram. The cells are arranged according to their distance from the base of the terminal filament. Individual cell volumes were estimated from the maximal cross-sectional area, using the formula $V = \pi ab^2/6$ (where "a" is the major axis and "b" the minor axis of the cross sectioned cell). The single cells in the anterior fifth were about twice the volume of those in the posterior four-fifths of the germarium. A wide range in cell volumes existed throughout the germarium. The mean volume for all of the 141 cells was 221 μ^3 with a standard error of 20 μ^3.

Fifty-four per cent of the cells in the sectioned *fes* germarium were not connected to any other cell. Such single cells were found throughout the germarium, and they sometimes contained spindle remnants, but lacked all trace of ring canals. Several unusually large single cells near the base of the terminal filament were probably stem line oogonia or cystoblasts. Most of the single cells were smaller and were similar in size to wild type cystocytes.

The remaining forty-six per cent of the cells in the *fes* germarium were in clusters containing two, three, four, or six cells. In wild type germaria, cystocyte clusters contain only two, four, eight, or sixteen cells. Clusters with fewer than sixteen cells are found only in the anterior region of the germarium, and the mean volume of the cystocytes decreases as the number per cluster increases (Koch and King, 1966). In the *fes* germarium, clusters were found in all regions, and the volume of the individual cells in a cluster did not decrease as the number of cells in the cluster increased. Two *fes* clusters with the same number of cells may contain large or small cells, and a considerable range in cystocyte sizes was also found within a single *fes* cluster.

In the wild type sixteen cell cluster, one quarter of the cells are located at branching points in the chain and have three or four ring canals. The plane of each cystocyte division is oriented so that one cystocyte retains all of the old ring canals. The hypothesis has been proposed (Koch, Smith and King, 1967, see their Fig. 7, and Koch and King, 1969) that in cystocytes both mother and daughter centrioles detach from the cell membrane, and migrate 90° in opposite directions until they are equidistant from all previously formed ring canals. Since this process is assumed to be repeated, the spindle axis is always oriented perpendicular to the spindle axis from the previous division. It appears that *fes* cystocytes have the normal ability to form such branching chains of cells (King, 1969b).

Wild type ring canal rims gradually enlarge, thicken, and accumulate on their inner surface a deposit which differs cytochemically from the rim itself (Koch and King, 1969). The rims of *fes* ring canals remain thin and delicate with little or no internal coating (Fig. 3). In the branching six cell clusters seen in the reconstructed *fes* germarium all of the canal rims had a similar appearance.

A pair of large nurse cells was found in the posterior region of this *fes* germarium (Fig. 2). Nurse cells have occasionally been observed in *fes* germaria under the light microscope (Koch and King, 1964). Migration of *fes* cystocytes through the germarium is evidently so abnormal that some cells remain there long enough to differentiate as nurse cells. A group of eleven single cells and two pairs of cells, adjacent to the nurse cells, were in various stages of degeneration. Areas of cystocyte degeneration are found occasionally in whole mounts of *both fes* and

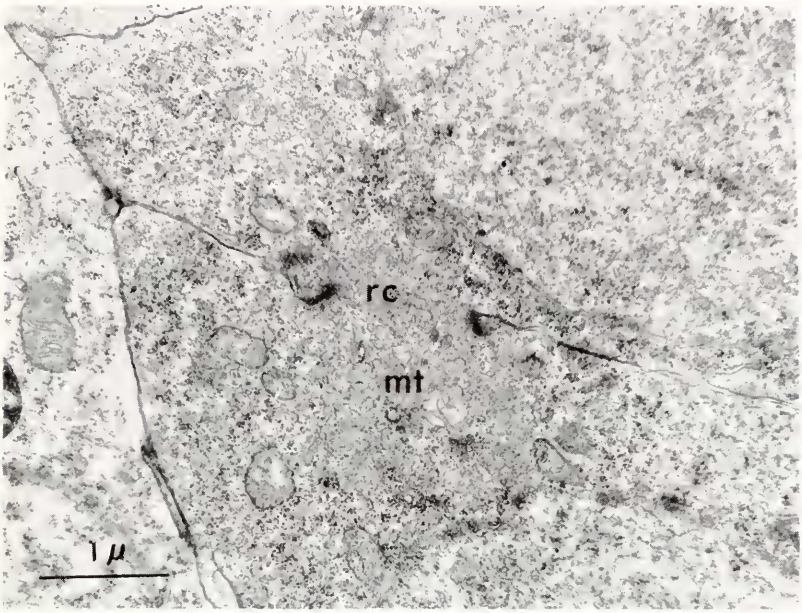

FIGURE 3. A ring canal (rc) found in the posterior half of the *fes* germarium. The canal rim has not undergone the growth and accumulation of an internal coating which are characteristic of wild type ring canals. Contrast with Koch and King (1969, their Fig. 5B). Microtubules (mt), which may represent remnants of the spindle, reside in and along side the canal.

wild type germaria, and therefore they are not a characteristic of the *fes* mutation. Cells with two and three nuclei were also found in the *fes* germarium (Fig. 2, m). According to Smith and Murphy (cited in King, 1969b), forty per cent of the cells were binucleate in *fes* chambers containing only nurse cells and oocytes.

The terminal filament, tunica propria, and the sheath of squamous cells covering the anterior region of the *fes* germarium do not differ from wild type. However, the centripetal migration of profollicle cells into the midregion of the germarium did not occur (compare Figs. 1A and B). Previous light microscopic studies of the *fes* germarium by Koch and King (1964) have shown the same general picture of a tightly packed mass of cystocytes that are not separated into regions by profollicle cells. The profollicle cells normally separate the actively dividing cells from differentiating cystocyte clusters (Koch and King, 1969).

At the posterior end of the germarium a cyst containing about fifty cells had formed. The most posterior follicle cells were cuboidal. The follicle cells at the junction of the cyst and the rest of the germarium were columnar. Although this cyst contained three times as many cells as a normal chamber, the follicle cells had not migrated between the cystocytes to form a stalk separating the cyst from the rest of the germarium. Koch and King (1964) have shown that a *fes* germarium takes about thirty hours to produce a chamber, whereas the wild type germarium takes a minimum of twelve hours. It follows that *fes* cysts separate from the germarium with great difficulty.

The reconstructed *fes* germarium was atypical in that it was not much larger

than the average wild type germarium. The *fes* germaria observed in the subsequent light microscopic studies were usually about sixty per cent broader at their widest point, and contained more cells than the reconstructed germarium.

Studies on dividing germarial cells

The frequency and position of metaphase figures, and the number of simultaneously dividing cells in a cystocyte cluster were tabulated from drawings of hundreds of Feulgen-stained *fes* and wild type germaria. Most of the following data was taken from germaria pretreated with 1×10^{-4} M colchicine four hours before sacrifice. Colchicine-pretreated germaria contained four to five and a half times as many metaphases as did uninjected controls (Table I).

Stem cell and cystoblast divisions were found in the most anterior fifteen microns of wild type germaria. Approximately equal numbers of clusters of two, four and eight metaphase figures were distributed throughout the anterior thirty microns of the wild type germarium (Fig. 4). Groups with intermediate numbers

TABLE I

The average number of metaphase figures per germarium in fes *and wild type ovaries raised at 18° and 25°* C

Phenotype	Temperature	Colchicine	Germaria examined	Metaphase figures/ germarium
+	25° C	no	697	0.54
+	25° C	yes	475	2.35
+	18° C	no	230	0.44
+	18° C	yes	259	1.71
fes	25° C	no	244	1.19
fes	25° C	yes	221	6.35
fes	18° C	no	127	1.17
fes	18° C	yes	310	6.45

of metaphases were not found. The situation in *fes* germaria was very different. Here groups with abnormal numbers of metaphases (3, 5, 6, 7, 9, 10, and 11) were observed, and the number of single metaphase and pairs of metaphases was much larger than normal (Fig. 4).

In the wild type germarium the cells in the sheath surrounding region one apparently divide infrequently, if at all, since mitoses have never been observed. Dividing profollicle cells were seen in a region about thirty microns posterior to the terminal filament. Here the profollicle cells begin to migrate between newly formed sixteen cell clusters, separating them from region one. Metaphase figures in dividing profollicle cells are more compact and oval in contour than those characterizing cystocytes.

More than twice as many dividing cells were found in the average *fes* germarium than in wild type. About half of these *fes* metaphases were seen in the posterior region of the germarium. Similar frequencies of metaphases were found in *fes* at both 25° and 18° C (Table I), whereas the frequencies of metaphases in wild type germaria were lower at the lower temperature in both the control and the colchicine

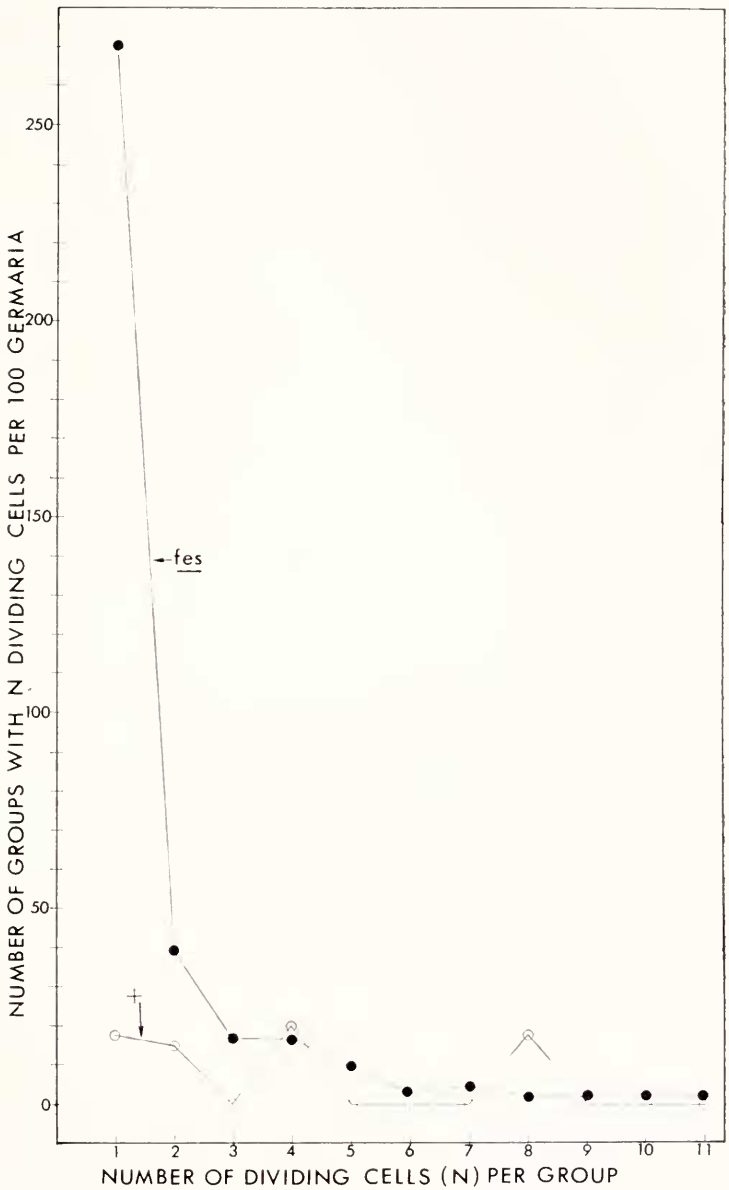

FIGURE 4. The distribution of groups with N dividing cells in germaria of colchicine-treated, wild type and *fes* females at 25° C.

series. If stem cells and cystoblasts are produced at about the same rate in *fes* and wild type, about half of the metaphases in *fes* germaria must be due to supernumerary divisions. We conclude that the average *fes* cystocyte undergoes one additional cycle of division at either temperature before leaving the germarium.

At 18° C and 25° C, thirty-eight per cent and forty-two per cent of the metaphases were in single cells. Most of the clusters contained only a few sister cells. Flies reared at 18° C had fewer single metaphases, and more large clusters. The difference between the pattern at 18° C and 25° C was significant at the 1% level using a chi square test. Single metaphases and clusters of two to eight dividing cells were found throughout the *fes* germarium. Since there are clusters with more than four dividing cells in the anterior half of the *fes* germarium, at least some clusters have undergone the normal number of divisions in this region. Ninety-two per cent of the metaphase figures in clusters of more than eight were found in the posterior half of the germarium. These clusters must have been undergoing supernumerary cycles of division.

The germaria from *fes* females are usually longer, broader, and have blunter tips than normal germaria. Geometric estimations of the volumes of both types of germaria were made, using the average dimensions from fifty drawings of whole mounts of *fes* and + germaria. The volume of the anterior five micron segment was calculated from the formula for the volume of a spherical segment [$V = \pi H^2/3$ (3R-H), where H is five microns, and R is the radius of the germarium five microns posterior to the base of the terminal filament]. The remainder of each *fes* or + "average" germarium was divided horizontally into a series of five micron segments. The volume of the frustrum of a right circular cone [$V = \pi H/3$ ($R^2 + r^2 + Rr$), where H is five microns; R, the radius at the larger end of the frustrum; and r, the smaller radius].

The wild type germarium has approximately one hundred twenty cells in regions one and two, excluding mesodermal cells (Smith and King, 1968). By dividing the average volume of a *fes* germarium by the average volume of one of its cells, we can estimate that there are approximately two hundred fifty cells inside of that region of the *fes* germarium equivalent to regions one plus two in the wild type germarium. The mean cell volume for the cells in the anterior fifth of the *fes* germarium was 329 μ^3, which is similar to the mean volume (311 μ^3) for cells anterior to the sixteen cell clusters in a reconstructed wild type germarium (Koch and King, 1966). Most of the cystocytes in wild type germaria are in region two, where they begin differentiation and stop growing temporarily. The cystocytes in each of these sixteen cell clusters have mean volumes of 90 μ^3 (Koch and King, 1969). Most *fes* cystocytes do not begin to differentiate after four cycles of division. Although there was a wide range in the sizes of individual cells, the mean volume for cells in the posterior four-fifths of the reconstructed *fes* germarium was 170 μ^3. This value is intermediate between the size of wild type cells in eight and sixteen cell clusters. It follows that *fes* cystocytes behave abnormally in that they continue to grow and divide in the posterior region of the germarium. As a result the average *fes* germarium contains more cystocytes than the wild type germarium, and most of these cells are larger than normal.

The anterior region of the *fes* germarium cannot be compared directly with region one of a normal germarium for two reasons. First, the actively dividing cells are not separated from older cystocytes by profollicle cells; and second, the *fes* germarium is abnormally broad and contains more cells than normal even at its anterior tip. Stem cells, cystoblasts, and cystocytes undergoing the normal number

of divisions reside in the anterior region of the *fes* germarium, and it probably also contains a large number of cells that have completed the normal number of divisions.

The number of metaphase figures in each five micron segment of germaria from *fes* females was determined, and the number of metaphases in each five micron segment was divided by the volume of each segment. These data demonstrated that the frequency of division was highest in the anterior region and decreased toward the posterior region of the *fes* germarium. Thus, although *fes* cystocytes undergo supernumerary divisions, they are not capable of dividing continuously at the rate characteristic of the initial cystocyte divisions. The maximum value in the anterior region ranged from 2.7 to 2.9 metaphases per 100 μ^3 per one hundred *fes* germaria. Comparable values for region one in wild type were 3.6 at 18° C, and 4.3 at 25° C. Although the frequency of division per unit volume is less in *fes* than in wild type, this difference probably reflects the presence of "old" cystocytes that have ceased dividing in the anterior region, rather than a longer intermitotic interval for *fes*.

Since a comparison of the division rates on the basis of volume or cell number does not take into account the failure of *fes* profollicle cells to segregate the "young" cystocytes that are in the initial mitotic cycles from the mitotically inactive, "old" cystocytes, a comparison was made of the frequencies of metaphases in equal lengths of *fes* and wild type germaria. If the number of metaphases in a 30 μ long, anterior region of a wild type germarium and the number in the same length of a *fes* germarium are compared, there are 1.3 times as many divisions found in the case of *fes*. Assuming that *fes* cystocytes during their initial four cycles of division have moved no further from the apex of the germarium than has a normal cluster undergoing the same number of divisions, then there may be no difference between the initial division rates for *fes* and wild type.

The behavior of profollicle cells in the fes *germarium*

Five different classes of abnormal germaria have been described in *fes* ovaries (Koch and King, 1964). These variations from the normal germarial morphology are due primarily to abnormalities in the migration of *fes* profollicle cells. Profollicle cells may invade the posterior region of the germarium at several points without splitting off a chamber. Large tumorous masses may remain at the base of the germarium for prolonged periods of time, or tumors may separate partially from the *fes* germarium before the follicle cells have undergone a centripetal migration. The abnormal migration of follicle cells at the base of a *fes* germarium usually results in a vitellarium which contains chambers with abnormal follicular envelopes and deformed interfollicular stalks. We believe that the abnormal growth and migration of the profollicle cells in most *fes* germaria is a consequence of the abnormal clusters, rather than a direct effect of the mutation.

In wild type germaria, metaphase figures were first seen in profollicle cells in the same region where these cells begin to send protoplasmic strands between newly formed sixteen cell clusters. Profollicle cells divide infrequently, if at all, in region one. Therefore a stimulus is provided for both migration and division in region two of a normal germarium. Metaphase figures in profollicle cells were not seen in the midregion of the *fes* germaria, which usually lack centripetally migrating profollicle cells. We suggest that the centripetal migration of profollicle

cells may require either the presence of a cluster containing a minimum number of cells or a change in the properties of the plasmalemma of cystocytes which coincides with the termination of their division or the beginning of their differentiation.

Metaphase figures were frequently observed in the cuboidal follicle cells in region three of the wild type germarium and at the posterior end of the *fcs* germarium. As we pointed out earlier, the separation of cysts from the *fcs* germarium is abnormally slow and inefficient. Apparently the organized growth of follicle cells in region three depends on the separation of clusters by profollicle cells in region two. Both the delayed formation of follicles and the continuing division of cystocytes cause the *fcs* germaria to become abnormally large.

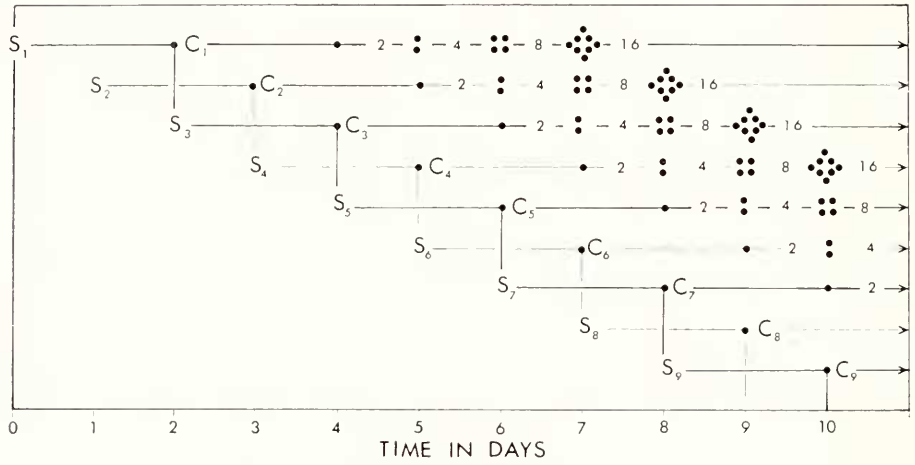

FIGURE 5. A graphic model of the functioning of a wild type ovariole. The ovariole contains two stem line oogonia (S_1 and S_2). These divide once every two days, but are out of phase by one day. Mitosis of a stem cell generates another stem cell and a cystoblast (C). This divides to produce (2) first generation cystocytes. When these divide a pair of metaphases are seen and (4) second generation cystocytes are formed. When these divide a cluster of four metaphases are seen and (8) third generation cystocytes are formed. When these divide a cluster of eight metaphases are seen and (16) fourth generation cystocysts are formed A steady state is generated in which we expect to find metaphases distributed among singles and clusters of 2, 4, and 8 in a 2:1:1:1 ratio. Stem cells, cystoblasts, and first, second, third, and fourth generation cystocytes are distributed in a 2:2:1:1:1:1 ratio.

DISCUSSION

Brown and King (1962, 1964) have presented evidence for the presence of stem line oogonia in each germarium of *Drosophila melanogaster*, and Koch and King (1966, their Fig. 2) have determined the distribution of single cells and clusters of 2, 4, 8, and 16 cystocytes in the wild type germarium. A model of the functioning of the normal germarium is presented in Figure 5. Here we assume that the fly is producing one mature egg per ovariole per day. The steady state system illustrated predicts a distribution of oogonia and cystocytes similar to that found by Koch and King, and it also predicts that the daily production of germ line metaphases should be 2 singles: 1 "twin": 1 "quadruplet": 1 "octuplet."

The experimental procedure employed in our colchicine experiments may have resulted in the collection of all division figures produced in the germaria of the injected wild type *Drosophila* during a four hour interval. The number of single metaphases and of groups of 2, 4, and 8 was about 18 per 100 germaria for each of the four categories. By multiplying by 6 and dividing by 100, we get the average value for each of the four metaphase classes, namely 1.08 per germarium per day. Since the observed value for the number of singles is one half the expected value, we conclude that the time spent during mitosis is shorter for single cells than for interconnected cells. On the other hand, the observed distribution of single cells and clusters of 2, 4, and 8 cystocytes (Koch and King, 1966) can best be explained, if one assumes that cells that double their birth size before dividing spend a longer time between divisions than do cystocytes.

The above calculations fit the expectations for a *Drosophila* female producing one egg per ovariole per day. However, it may take some time for the colchicine to reach the germarial cells in an effective concentration, and therefore the actual time during which all metaphases were collected may be considerably less than four hours. If we assume that only half of the cells, which divided during the period between colchicine injection and sacrifice of the fly were recorded, then the average value for each of the four metaphase classes would rise to 2. This would correspond to the situation where the female produced two eggs per ovariole per day, the maximum rate observed for this species (see King, 1970, page 50).

Another explanation can be put forth to account for the deficiency in the number of single metaphases found in germarial region 1. We assumed in our calculations that single cells and interconnected cells are dividing throughout the day. If single cells divide more often at night and interconnected cells divide more often during the day, then cystocyte metaphases would have been selected, since experiments were generally carried out between 10 AM and 4 PM.

One cannot argue that the deficiency in the number of single metaphases results from a difference in sensitivity of single cells and interconnected cells to colchicine, because Grell (1967, her Fig. 16) also found a 1:1:1:1 ratio of singles, twins, quadruplets, and octuplets in the ovaries of untreated pupae.

The first chamber in the vitellarium of *Drosophila melanogaster* is surrounded by an envelope containing approximately 80 cuboidal follicle cells (King and Vanoucek, 1960). These are derived from the population of profollicle cells in the germarium. Since under optimal conditions a female oviposits two eggs per day, 160 germarial profollicle cells are lost daily. It follows that the germarium must contain mitotically active, profollicle cells that serve to replenish those lost. Mitotically active, stemline oogonia reside in the anterior region of the germarium (Fig. 1A, *sc*). Our colchicine experiments demonstrate that mitotically active, profollicle cells reside in a region about 30 μ behind the terminal filament. These cells are thought to be of mesodermal origin (King, Aggarwal, and Aggarwal, 1968), and they presumably generate the cells that envelope the cystocyte cluster before it enters the vitellarium.

In *Drosophila melanogaster* the earliest step in the formation of a normal cystocyte cluster is a series of divisions which produces a branched chain of sixteen cells. We conclude that the *fes* mutation affects these divisions, since the patterns of intercellular connections and the numbers of sister cells per cluster were often

abnormal in the germarium we reconstructed. The same holds true for the sister cells in tumorous and "nurse cell" chambers in the vitellarium (Koch and King, 1964). The patterns formed by the interconnected cells in the *fes* ovariole are generally asymmetric (see Koch, Smith, and King, 1967, their Fig. 9), and some of the cells normally produced during a cycle of division are missing. Therefore we conclude that the mutation affects the cystocytes in a cluster independently and at random.

Some *fes* clusters contain cells formed during the supernumerary division, and some of the cells normally found in a cluster are missing. Usually no cell with four ring canals, and consequently no oocyte, is present (King, 1969b). Clusters with more than the normal number of cells would be expected because of the high frequency of supernumerary division; however, most *fes* clusters contain very few, usually only two or three cells, and approximately 40 per cent of the germarial cells are not part of a cluster.

The abnormal patterns of interconnections found in *fes* clusters suggest that the primary effect of the *fes* mutation is the elimination of some of the cells within a cluster. This elimination occurs at random and effects any cells in a cluster independent of its neighbors, causing asymmetric deviations from the normal pattern of connections. As a consequence of this primary abnormality, the remaining cells may not stop dividing after the normal number of mitotic cycles. It is assumed that the spindle axes rotate normally, and that ring canals are stable, once they are formed. There are two simple ways in which a cell may be eliminated from a cluster. Either some cells fail to divide, or the nuclear divisions are normal, but cytokinesis is complete.

Small clusters with abnormal numbers of cells would be produced if some of the cells failed to divide in each mitotic cycle (see Fig. 6II). Although failure of cystocyte mitosis will account for the pattern of cell interconnections within a *fes* cluster, it cannot explain the action of the *fes* mutation for the reasons given below.

Normally the number of cystocystes doubles with each cycle of division. If some divisions fail to occur, the average cystocyte will produce $(2 - r)$ cells and these will continue to divide forming $(2 - r)^2$, $(2 - r)^3$, and finally $(2 - r)^4$ cells. Here r is the probability that division does not occur, and we assume the value remains constant at each cell division. According to this argument *fes* germaria should produce fewer new cells and the difference between the number of new cells generated in *fes* and wild type would be magnified with each division cycle. The *fes* germarium, however, does not show a reduced production of new cells.

All of the unconnected cells are stem cells or undivided cystoblasts according to the explanation which bases the action of the *fes* mutation on a failure of mitosis. If cystoblasts are produced even at the maximum rate of two per day, it is impossible to account for the 69 unconnected cells found in the reconstructed germarium, which was fixed 48 hours after the insect eclosed. Finally, most of the single cells in the *fes* germarium were below the volumes characteristic of either stem cells or cystoblasts. Instead the unconnected *fes* cells had the same range of volumes normally seen in cystocytes.

Normal cystocytes remain connected because cytokinesis is incomplete. A second hypothesis for the *fes* mutation is that some mutant cytoblasts and cystocytes undergo complete cytokinesis. It is assumed that the failure to form a ring

canal joining sister cystocytes is a random process, which may independently effect any dividing cell during any cycle of division. Since cytokinesis, rather than nuclear division, is abnormal, the total number of cells would double in each cycle

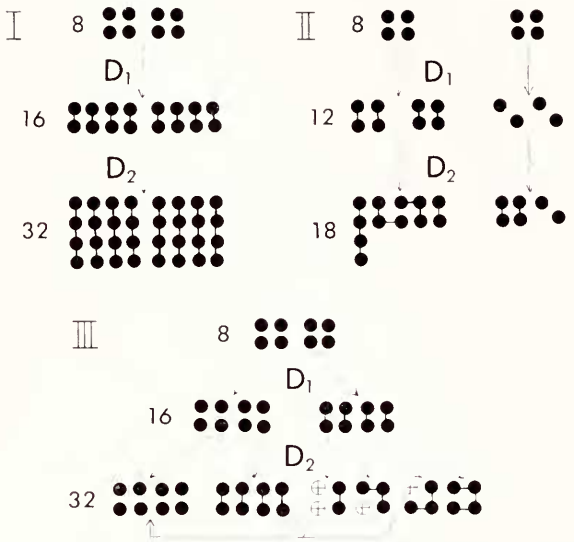

FIGURE 6. A diagrammatic comparison of the first two cycles of cystocyte division in wild type germaria (I) and in mutant germaria (II and III). In mutant model II the division of half of the cystocytes is suppressed, but those that do undergo mitosis, form canals in the normal way. In mutant model III all cystocytes undergo mitosis, but half of the cells undergo complete cleavage and the other half form ring canals during each division cycle. D_1 and D_2 represent the first and second division cycles. Each diagram begins with eight cystoblasts. (I) In the wild type germarium each cystoblast divides once forming a two cell cluster. Both cells in each cluster divide a second time producing a chain of four cells connected by ring canals. The final number of cells is 32. (II) In the mutant germarium half of the cells fail to multiply in each cycle, and the 2 unconnected cells present in the final population are those cystoblasts which failed to multiply on both occasions. Clusters of three or four interconnected cells are formed, if one or both of the cells in a two cell cluster divided during the second cycle. Although this scheme produces the small, abnormal clusters found in *fes* germaria, it cannot generate large numbers of unconnected cells. Note that the total number of cells after each division cycle is less than in wild type. (III) In this model half of all cystocyte mitoses in the mutant are followed by complete cytokinesis. Cystoblasts form either a two cell cluster, or two unconnected daughter cells. These unconnected daughter cells may in turn form two cell clusters, or additional unconnected cells. If one or both of the cells in a two cell cluster forms a ring canal during the next division, a three or a four cell cluster is made. As in wild type (I), the total number of cells doubles during each division cycle. Unlike model II, unconnected daughter cells are generated from joined cystocyte pairs during D_2. These single cells are symbolized by $\oplus$, and they join the pool of single cells generated by cells that were never part of a cluster. Since some unconnected cells may start new clusters, a single cystoblast may eventually give rise to more than one cluster. In the example shown, 8 cystoblasts produce (in two cycles of division) : 1 four-cell cluster, 2 three-cell clusters, 5 two-cell clusters, and twelve unconnected cells. During the second cycle of division some cells are budded off clusters, and these single cells can be treated mathematically in two different ways. In the algebraic model in Figure 7A we assume that they continue to multiply, but only a given fraction of the daughter cells remain connected. In Figure 7B we assume the cells continue further multiplication, but daughter cells invariably undergo complete cleavage.

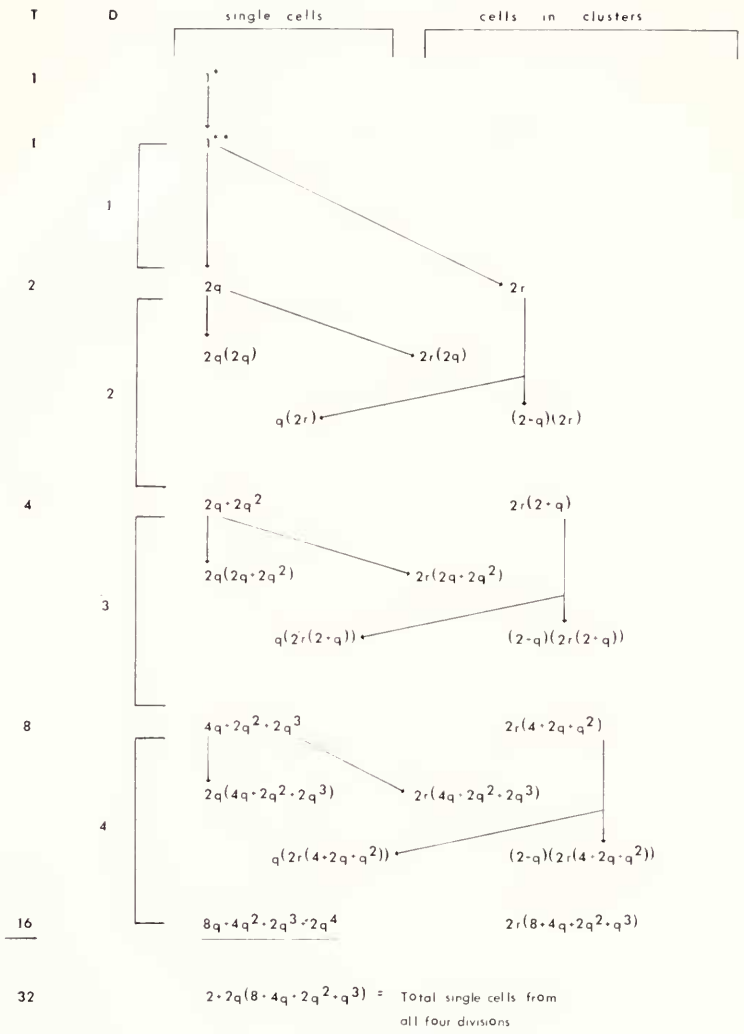

FIGURE 7A

FIGURE 7. A pair of algebraic models which generate the probable frequencies of single cells and of cells in clusters, if a constant fraction of cells undergo complete cytokinesis in each division cycle. Here q is the probability that a connected cell will undergo complete cytokinesis. r is $1-q$, the probability that a cell will form a ring canal. 1* represents the stem line oogonium. 1** represents the cystoblast. T gives the total cells at the end of each division, and D gives the cystocyte division cycle. Single cells in the left column produce additional cells, if they divide completely; and two cell clusters, if they form ring canals. When the cells in a cluster divide, the new daughter cells either remain attached by ring canals or separate from the cluster as additional single cells. In (A) the single cells produced from a cluster are added to the single cells which have never been part of a cluster in the left column. All single cells are assumed to have an equal probability of forming a two-cell cluster during subsequent divisions. In (B) the single cells split off from a cluster form a separate pool of cells in the

of division. However, unlike wild type cystocytes, the cells generated will be found to be either unconnected or connected in abnormal small clusters (see Fig. 6 III).

The complete cytokinesis hypothesis provides two sources of single cells: they may be the direct descendants of the original cystoblast, or they may be budded off from the cystocytes of a cluster. Since these two groups of single cells may or may not have the same potential ability to start new clusters, there are two variations of this hypothesis.

In the first variation, all single cells, whether they are direct descendants of the cystoblast or of cystocytes in a cluster, have the same probability of forming a ring canal at the next mitosis. The exchange of cells between clusters and the pool of single cells is shown in Figure 7A.

In the second variation of the complete cytokinesis hypothesis, the cystoblast, and its direct descendants, which have never been part of a cluster, are the only single cells with the capacity of forming canals. A second pool of single cells is formed by the cystocytes that have been split off of clusters. These cystocystes retain the ability to divide, but not the ability to form ring canals. The predictions as to the relative numbers of both types of single cells and of cells in clusters are shown in Figure 7B. In this variation the percentage of cells belonging to the pool of single cells unable to form clusters increases with each cycle of division.

The relative frequency of clusters containing differing numbers of cystocytes in the germarium can be predicted using the binomial expansion. This process is repeated for each size cluster, and for each cycle of division (see Fig. 8). Clusters with the same number of cells may have different internal patterns, and

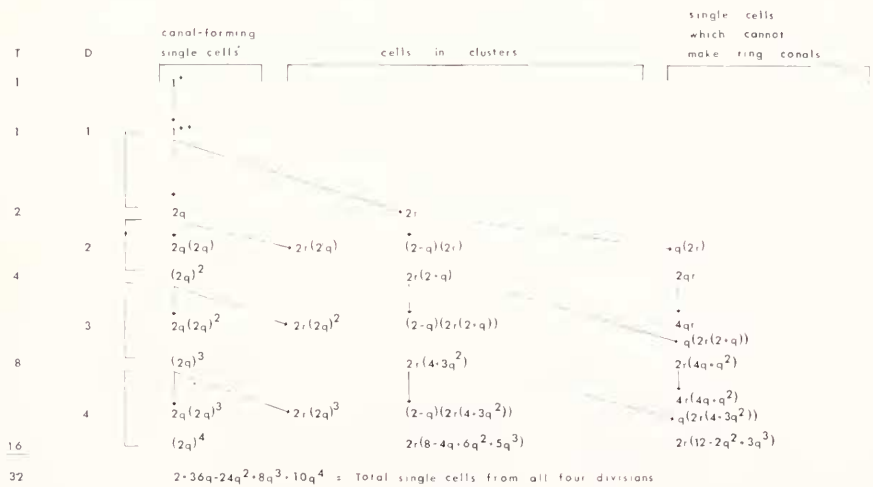

FIGURE 7B

right column. These single cells are assumed capable of further division, but incapable of forming ring canals in future divisions. In both diagrams the probable number of cells from all sources at the end of a division cycle is summed in each category. Arrows run from this sum to the probable distribution of these cells after a further cycle of division.

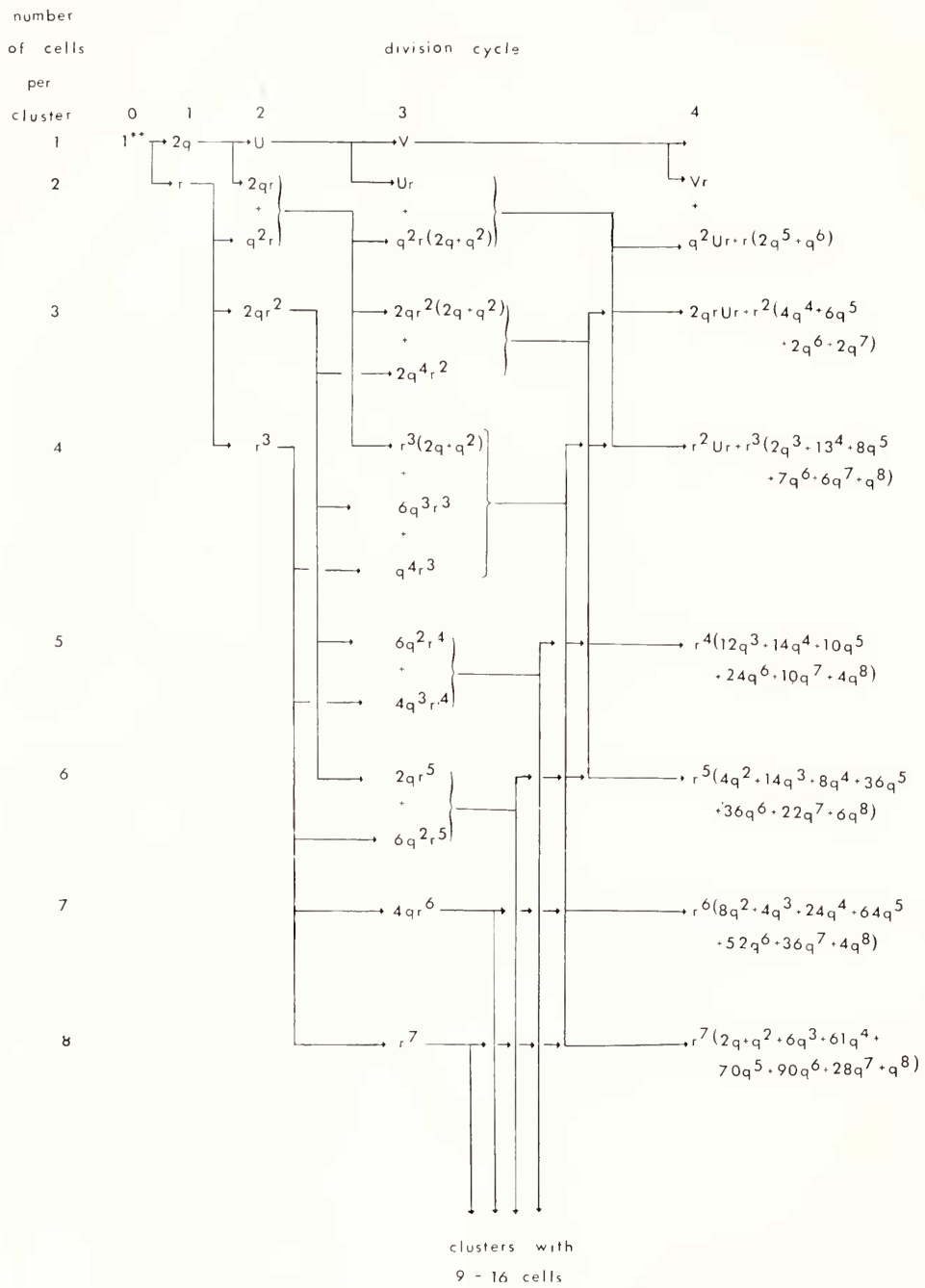
number
of cells
per
cluster
division cycle
0
1
2
3
4
1
2
3
4
5
6
7
8
1**
2q
U
V
r
2qr
+
q2r
Ur
+
q2r(2q+q2)
Vr
+
q2Ur+r2(2q5+q6)
2qr2
2qr2(2q+q2)
+
2q4r2
2qrUr+r2(4q4+6q5
+2q6+2q7)
r3
r3(2q+q2)
+
6q3r3
+
q4r3
r2Ur+r3(2q3+13q4+8q5
+7q6+6q7+q8)
6q2r4
+
4q3r4
r4(12q3+14q4+10q5
+24q6+10q7+4q8)
2qr5
+
6q2r5
r5(4q2+14q3+8q4+36q5
+36q6+22q7+6q8)
4qr6
r6(8q2+4q3+24q4+64q5
+52q6+36q7+4q8)
r7
r7(2q+q2+6q3+61q4+
70q5+90q6+28q7+q8)
clusters with
9 - 16 cells

FIGURE 8.

may be formed during different cycles of division. We assume that exactly one additional cycle of division occurred in the *fes* germarium. Since the number of metaphases in a cluster indicates the number of cells formed in the previous division, the additional cycle of division in *fes* shows the configurations which were produced during the fourth cystocyte division. The predicted relative frequency for each size cluster was based on the sum of the predictions for all cycles of division, assuming the four cycles to be equally represented in the germarium. A test was calculated to determine which variation of the model gave the best fit to the overall relative frequencies of clusters of different sizes observed in the germarium.

Since single cells were the largest group observed, a value for q, the probability of complete cytokinesis, was calculated using the observed ratio of single cells to total cells. Single cells make up 38.4% of the total at 18° C, and 41.8% of the total cells at 25° C. Assuming equal representation of the four division cycles, there should be $2 + 2q(8 + 4q + 2q^2 + q^3)$ single cells for every 32 cells according to the first variation, and $2 + 36q - 24q^2 + 8q^3 + 10q^4$ single cells for every 32 cells according to the second variation of the complete cytokinesis hypothesis (see Fig. 7). Values of q were approximated by setting the observed ratio equal to the predicted ratio of single cells to cells in clusters and running a trial series of calculations to estimate q to three significant digits. Four values for q were approximated, one for each variation for the data at 18° C and at 25° C. These values of q were used to predict the number of clusters with two to eight cells in *fes* germaria from the two temperature series. There were too few observations of large clusters to warrant continuing the calculations.

In Table II the four sets of predictions are compared with the data collected from the colchicine-treated *fes* germaria. A chi-square value was calculated from the deviations between each set of predictions and the appropriate set of data. The number of clusters predicted by the first variation of this hypothesis was very highly significantly different from the observed data. The ratio of single cells to

FIGURE 8. Expressions for the frequencies of clusters with two to eight cells, after one to four cycles of division, starting from a single cystoblast (1**). The first row shows the frequencies of canal-forming single cells, and corresponds to the first column of Figure 7A or 7B. This frequency differs after the second division cycle according to which model is considered, and is represented by U or V after two or three divisions, respectively. U is $2q + 2q^2$ in the first model (Figure 7A) and $(2q)^2$ in the second model (Figure 7B). V is $4q + 2q^2 + 2q^3$ in the first model, and $(2q)^3$ in the second. The probability of complete cytokinesis is q, and $r = 1 - q$ is the probability that a cell will form a ring canal. Branching arrows run from the frequency of each given cluster to its corresponding frequency contributions in clusters of equal or greater sizes in the following division cycle. For example, after the second division cycle there are on the average $2qr + q^2r = r(2q + q^2)$ two cell clusters, and these lead to clusters of two, three or four cells at the third cycle in proportion of q^2, $2qr$ and r^2, respectively. Hence arrows lead from $2qr + q^2r$ (two-cell clusters at division cycle 2) to the proportionate terms $q^2r(2q + q^2)$, $2qr^2(2q + q^2)$ and $r^3(2q + q^2)$ at the third cycle. It should be kept in mind that the frequencies shown here are for clusters, rather than for cells in clusters as in Figure 7. Thus to obtain the frequency of cells in clusters of a given size it is necessary to multiply the cluster frequency by the number of cells in the cluster. For example, the total number of cells after two division cycles is found by summing $1(U) + 2(2qr + q^2r) + 3(2qr^2) + 4(r^3)$. The total is 4 under the first model and $4 - 2qr$ under the second, as it should be after two cell divisions. The difference is just the number of single cells that cannot form ring canals under the second model. These are not shown in this diagram.

cells in small clusters was too low with this set of calculations. The second variation of the hypothesis predicted values that were not significantly different from the observed data. Therefore, assuming equal representation of all four division cycles, the hypothesis that (1) some of the *fes* cystoblasts and cystocytes undergo complete cytokinesis and (2) that cells, which are split off cytocyte clusters, can only undergo complete cytokinesis is in best agreement with the data.

In the older literature many accounts have been published describing in various insects situations where sister germ cells are joined by canals (see review in King and Akai, 1971). A canal system similar to that connecting *Drosophila* cystocytes

TABLE II

Comparison of the number of groups of one to eight cells observed in colchicine-treated fes *germaria, with the number of groups predicted in each category by both variations of the "complete cytokinesis" hypothesis. The probability that a* fes *cell will undergo complete cytokinesis is given by* q

	Cells per group	Groups observed	Groups predicted 1.	Groups predicted 2.
18° C	1	768	768.4	768.5
	2	127	225.0	120.0
	3	45	66.8	46.8
	4	40	48.2	50.0
	5	25	20.5	19.6
	6	13	15.6	21.0
	7	23	9.5	15.6
	8	11	5.6	10.6
			$q = 0.488$	$q = 0.356$
			$\chi^2 = 77.34$	$\chi^2 = 10.66$
			$p < 0.0001$	$p = 0.15$
25° C	1	588	587.6	589.0
	2	92	162.2	89.8
	3	37	48.4	36.7
	4	37	31.3	33.9
	5	21	13.5	14.6
	6	7	9.4	13.8
	7	12	5.4	9.5
	8	4	3.0	6.1
			$q = 0.527$	$q = 0.403$
			$\chi^2 = 47.67$	$\chi^2 = 8.37$
			$p < 0.0001$	$p = 0.3$

is found between sister spermatocytes in *Bombyx mori*. King and Akai have observed midbodies and contractile rings in partly cleaved sister cells (1971, their Fig. 3), and they suggest that material from the midbodies somehow crosslinks the component fibrils of the contractile rings and prevents them from closing down further. Subsequently the midbody dissolves, and the "stabilized" contractile ring serves as the canal rim. We propose that the contractile ring also serves as the organelle about which the ring canal is elaborated in *Drosophila melanogaster* and that the product of the *fes* gene functions in the stabilization of the contractile ring. The values calculated for the frequency of complete cytokinesis for *fes* cystocytes were 0.356 for females reared at 18° C and 0.403 for females reared at 25° C.

At both temperatures wild type flies give zero values. We conclude that the product of the mutant gene is unstable, particularly at higher temperatures, and that it is successful in preventing the complete closure of the contractile ring only a fraction of the time. The "immature" appearance of each canal rim observed in the reconstructured germarium (Fig. 3) is in harmony with the above hypothesis.

In the germinal syncytia observed in *Drosophila melanogaster* and other species, all of the cells sharing a common cytoplasm divide in synchrony. Since the division of all such cells apparently is controlled as a unit, it is reasonable to suggest that a cue which causes one or more of the interconnected cells to differentiate will generate a sequence of reactions that terminates the mitotic activity of all other cells in the cluster. If the formation of two cells with four ring canals during the fourth cystocyte division is the cue for the differentiation of the pro-oocytes, their differentiation may terminate further mitosis among the other 14 sister cells. In *fes* germaria cystocyte divisions would continue indefinitely, since cells with four canals are generated so infrequently.

However, in *fes* ovarioles we do find clusters in which cystocytes have differentiated into nurse cells (which presumably are incapable of mitosis) even though the cluster lacks pro-oocytes. The cue for nurse cell differentiation may be related to the volume of the cells in question. In the normal germarium cystocytes do not double their volumes between divisions, and consequently the volume of each individual cell is reduced with each division. The average cell in a sixteen cell cluster is only one-fifth the volume of the original cystoblast. In *fes* the frequency of clusters of nurse cells in the vitellarium is about ten times greater for flies reared at 18° C than at 25° C (King, Koch, and Cassens, 1961). We know that in the germarium mitoses occur with equal frequencies at both temperatures (Table I). Perhaps lowering the temperature slows down the growth between divisions, so that after a few divisions a critical minimum volume is reached. This then serves as a cue which causes the cell to enter the nurse cell developmental pathway.

The authors are grateful for the useful criticisms of an early draft of this paper provided by Drs. L. T. Douglas, E. A. Koch, P. A. Smith, E. B. Spiess, N. E. Welker, and J. M. Whitten. Mr. David Calhoun checked the calculations and made useful suggestions as to the textual exposition of the mathematics. The illustrations were inked by Mr. E. J. Pfiffner. This research was supported by Public Service Grant 5TL GM903 and National Science Foundation Grant GB29279.

Summary

Females of *Drosophila melanogaster* homozygous for the autosomal, recessive gene *fes* are sterile, and their ovaries contain "tumorous" cysts that continue to grow mitotically and may eventually posses thousands of undifferentiated cells. To study the earliest steps in the formation of a *fes* "tumor" we determined the three dimensional interrelations of the cells in a single mutant germarium utilizing electron micrographs taken of serial ultrathin sections. This germarium contained a large number of unconnected cells and clusters made up of only a few interconnected cystocytes. The distributions of dividing cells in *fes* and wild type

germaria, some of which were treated with colchicine, were also studied. All of the cystocyte divisions take place in the anterior third of the wild type germarium. Here a few isolated metaphases were seen in stem line oogonia and cystoblasts, and the rest of the metaphase figures were found in groups of 2, 4, and 8 and presumably represented dividing cystocytes. Metaphases were found throughout the *fes* germarium. The number of isolated metaphase figures observed in mutant germaria was 15–20 times higher than in wild type. Metaphases were also found in groups. Clusters of two were twice as abundant in *fes* as in wild type, and clusters of four were equally abundant. Clusters of eight were seen about six times more often in wild type than in *fes*, but clusters of 3, 5, 6, 7, 9, 10, and 11 metaphases (which were never observed in wild type germaria) were found in *fes*. We estimated that the average *fes* cystocyte undergoes one supernumerary division before leaving the germarium.

We concluded that while all cystocytes undergo incomplete division in wild type germaria, a significant fraction of *fes* cystocytes undergo complete cytokinesis. An algebraic model developed from this hypothesis predicts the relative frequencies of single cells and clusters containing between 2 and 8 cells and enables us to calculate q, the probability that cystocytes will undergo complete cytokinesis. The predicted frequencies were not significantly different from those observed, and the hypothesis was also consistent with the observed rate of division found in the colchicine-treated ovaries and the patterns of cytocyte interconnections found in the reconstructed *fes* germarium. Germaria from *fes* females reared at 18° and 25° C gave q values of 0.356 and 0.403, respectively. The mitotic rates were the same at both temperatures. We conclude that the product of the *fes* + gene is required for the formation of a stable canal system and suggest that the product of the mutant gene is defective in this regard and thermolabile. The *fes* + substance may function to prevent the constriction of the contractile ring during cystocyte cytokinesis.

LITERATURE CITED

Brown, E. H., and R. C. King, 1962. Oogonial and spermatogonial differentiation within a mosaic gonad of *Drosophila melanogaster*. *Growth*, **26**: 53–70.

Brown, E. H., and R. C. King, 1964. Studies on the events resulting in the formation of an egg chamber in *Drosophila melanogaster*. *Growth*, **28**: 41–81.

Butterworth, F. M., D. Bodenstein and R. C. King, 1965. Adipose tissue of *Drosophila melanogaster*. I. An experimental study of larval fat body. *J. Exp. Zool.*, **158**: 141–154.

David, J., 1962. A new medium for rearing *Drosophila* in axenic conditions. *Drosophila Information Service*, **36**: 128.

Eakin, R. M., and J. A. Westfall, 1962. Fine structure of photoreceptors in the Hydromedusan, *Polyorchis penicillatus*. *Proc. Nat. Acad. Sci.*, **48**: 826–833.

Grell, R. F., 1967. Pairing at the choromosomal level. *J. Cell. Physiol.*, **70**:(1) 119–146.

King, R. C., 1969a. The hereditary ovarian tumors of *Drosophila melanogaster*. *Nat. Can. Inst. Monogr.*, No. **31**: 323–345.

King, R. C., 1969b. Control of oocyte formation of *female sterile (fes) Drosophila melanogaster*. *Nat. Can. Inst. Monogr.* No. **31**: 347–349.

King, R. C., 1970. *Ovarian Development in* Drosophila melanogaster. Academic Press, New York.

King, R. C., S. K. Aggarwal and U. Aggarwal, 1968. The development of the female *Drosophila* reproductive system. *J. Morphol.*, **124**: 143–166.

KING, R. C., AND H. AKAI, 1971. Spermatogenesis in *Bombyx mori*. I. The canal system joining sister spermatocytes. *J. Morphol.*, **134**: 47–56.

KING, R. C., AND D. BODENSTEIN, 1965. The transplanatation of ovaries between genetically sterile and wild type *Drosophila melanogaster*. *Z. Naturforsch.*, **20b**: 292–297.

KING, R. C., R. G. BURNETT AND N. A. STALEY, 1957. Oogenesis in adult *Drosophila melanogaster*. IV. Hereditary ovarian tumors. *Growth*, **21**: 239–261.

KING, R. C., E. A. KOCH AND G. A. CASSENS, 1961. The effect of temperature upon the hereditary ovarian tumors of the *fes* mutant of *Drosophila melanogaster*. *Growth*, **25**: 45–65.

KING, R. C., AND E. G. VANOUCEK, 1960. Oogenesis in adult *Drosophila melanogaster*. X. Studies on the behavior of the follicle cells. *Growth*, **24**: 333–338.

KOCH, E. A., AND R. C. KING, 1964. Studies on the *fes* mutant of *Drosophila melanogaster*. *Growth*, **28**: 325–369.

KOCH, E. A., AND R. C. KING, 1966. The origin and early differentiation of the egg chamber of *Drosophila melanogaster*. *J. Morphol.*, **119**: 283–304.

KOCH, E. A., AND R. C. KING, 1969. Further studies on the ring canal system of the ovarian cystocytes of *Drosophila melanogaster*. *Z. Zellforsch.*, **102**: 129–152.

KOCH, E. A., P. A. SMITH AND R. C. KING, 1967. The division and differentiation of *Drosophila* cystocytes. *J. Morphol.*, **121**: 55–70.

LINDSLEY, D. L., AND E. H. GRELL, 1968. *Genetic Variations of* Drosophila melanogaster. Carnegie Institute of Washington Publication, No. 627, Washington.

MOLLENHAUER, H. H., 1964. Plastic embedding mixtures for electron microscopy. *Stain Technol.*, **39**: 111–114.

REYNOLDS, E. S., 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.*, **17**: 208–212.

SMITH, P. A., AND R. C. KING, 1968. Genetic control of synaptonemal complexes in *Drosophila melanogaster*. *Genetics*, **60**: 335–351.